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DISTRIBUTION OF PHOTOTROPHIC THIONIC BACTERIA IN THE ANAEROBIC AND MICRO-AEROPHILIC STRATA OF MANGROVE ECOSYSTEM OF COCHIN *

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ABSTRACT

Dense population of epipelic photoautotrophic bacteria were found in the surface sediments during April 1984 to October 1984 in the mangrove ecosystem of Cochin. Pigment analysis by Spectrophotometric techniques revealed the structure and relative abundance of the populations. Maximum bacterio-chlorophyll 'a' was encountered in May (1650.6 mg/m^{-3}) whereas bacteriochlorophyll 'c' and 'd' were found to be more during April 1984 (1069.2 mg/m^{-3} and 510 mg/m^{-3} respectively). The bacterial pigments developed in the anoxic cultures in the present investigation showed absorption maximum at 662 nm, therefore it can be classified as 'Chlorobium chlorophyll 660'. The predominant bacteria were identified as members of the genera *Chloronema, Chromatium, Beggiatoa, Thiopedia* and *Leucothiobacteria*. Unidentified brown Chlorobiaceae were present. Maximal development of the population was found during April 1984. The distribution of oxygen, temperature salinity, pH and Eh profiles were determined. Phototrophic sulfur bacteria ranged from 4.2-19.4% of the total anaerobes isolated. The main factors determining the growth of green sulfur bacteria were light and high sulfide concentration whereas the growth of purple sulfur bacteria was mainly controlled by subdued light and low sulfide concentration during the experimental period.

INTRODUCTION

THE DISTRIBUTION of photosynthetic thionic bacteria is restricted by their requirements for both anoxic conditions and light (Fenchel and Blackburn, 1979 ; Pfennig, 1967) where light reaches shallow, marshy, anoxic waters. In some such systems the biomass of photosynthetic bacteria is often as large as the algal biomass (Cloern *et al.*, 1983, Indrebo *et al.*, 1979). The biomass of photosynthetic bacteria is frequently calculated from bacterial chlorophyll measured from the absorbance of 90% acetone extracts of particles (Takahashi and Ichimura, 1968, 1970 ; Caraco, 1986).

The production of sulfide due to sulfate reduction is the phenomenon widely reported in microbial ecosystems under anaerobic conditions (Skyring *et al.*, 1979 ; Sweeney and Kaplan, 1980). Besides their effects on the sulfur cycle thionic bacteria which transform sulfur or its compounds may influence pH, Eh, colour, carbonate content and oxygen tension in mangrove sediments due to their anaerobic thiogenesis.

It is now known that there are many species of morphologically and metabolically diverse sulphate-reducing bacteria which derive their energy by oxidation of simple compounds (Postgate, 1984 ; Skyring *et al.*, 1977 ; Widdel and Pfennig, 1981). Depending on growth rates and efficiency these microbes may produce 0.1 to 0.001 times their own weight of H_2S in 24 hours (Chambers *et al.*, 1975). The present paper is a comprehensive study of thionic bacterial activity in mangrove ecosystem.

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The study was aimed at understanding the distribution of bacterial parameters like total anaerobes, sulphur-reducers and green and purple sulphur bacteria and other environmental parameters like temperature, salinity, oxygen, pH and Eh in premonsoon and monsoon months and to understand the major factors controlling the photosynthetic reoxidation of sulphide and effect of these factors on thionic bacteria. An attempt was also made to estimate the bacterio-chlorophyll produced in anoxic cultures during the experimental period to understand the nature of the bacterio-chlorophyll-protein complex in these cultures and to calculate the biomass of photosynthetic bacteria. These bacteria contribute to the purification of organic waste solutions and metabolize diethyl nitrosamine, which is a carcinogenic, mutagenic and tetragenic substance. Also these bacteria fulfill a role in the sanitary purification of foul water and they have anti-viral properties. They are beneficial to suppress plant pathogenic micro-organisms in the mangrove vegetation. It is shown that phototrophic bacteria play an important role in the preservation of the mangrove environment (Kabayashi *et al.*, 1978).

Material and Methods

Study area : The bacteriological investigation was conducted in only one station for a period of 8 months from March to October, 1984, around Karuthedam near Cochin located between 9°55′N-10°5′N and 76°10′E-76°20′E (Fig. 1). The area is a typical tropical mangrove ecosystem with an abundant macrophytic flora constituted mainly by *Acanthus ilicifolius* and *Avicinnia officinalis*.

Parameters of study : Total anaerobes and sulphur reducers GSB and PSB were estimated from the sediment samples for a period of 8 months. Bacterio-chlorophyll (Bchl) was estimated from the cultures of GSB in all the months. Soil temparature, salinity, oxygen, Eh and pH were also investigated.

Collection of samples : Fortnightly sampling of soil from the station was done. Soil samples were collected from surface upto 5 cm depth using a mud corer aseptically into sterile polythene bags and plating was done within 18-30 hours of collection.

Redox potential : Redox potential was measured by an ELICO pH meter using platinum Eh electrode. Sediments with a redox potential above 300 mv were regarded as oxic ; sediments between 300 and 100 mv as suboxic and sediments below 100 mv as anoxic (Graf *et al.*, 1983). pH was measured by the same ELICO pH meter using standard and calom elelectrodes.

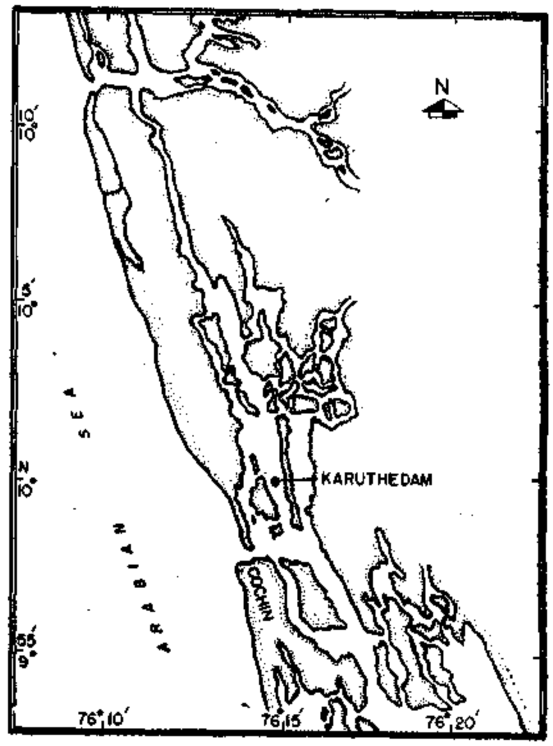

Fig. 1. Sampling Station (Karuthedam) in the mangrove region near Cochin.

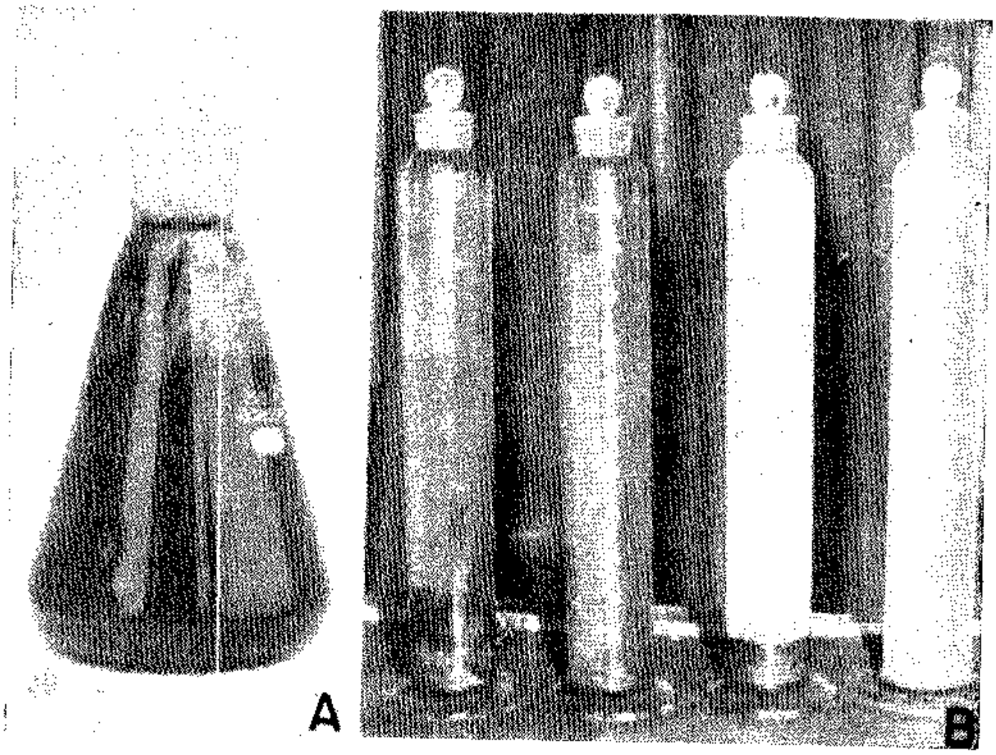

PLATE I. A. *Leucothiobacteria* showing sulphur deposits and B. Green sulphur bacteria producing H_2S in an anaerobic medium

Bacterial parameters : Approximately 1 g of the sediment was aseptically transferred to 99 ml sterile aged water in a conical flask and agitated in a shaker for uniform mixing. After thorough mixing one ml of the sample was used for pour-plating procedures.

The sea water medium (SWA) was used for total anaerobes. Sulphur-reducing anaerobes was isolated in sea water agar supplemented with 1% lead-acetate, 1% glucose and 0.1% sodium thioglycolleate. The plates were incubated in Mc Intosh Flide anaerobic Jar at RT for 7 days and then counts were made.

Culture conditions for photosynthetic bacteria : Green sulphur bacteria (GSB, Order Pseudomonadales, Sub-order Chlorobiineae, Family—Chlorobiaceae and Chloroflexaceae ; Trüper, 1976) was cultured in sterile aged sea water supplemented with 2% of Asparagin crystals and PSB (Family—Thiorhodaceae) was cultured by using sterile aged sea water and egg white 2 g/litre instead of Asparagin crystals. 1 g mud was aseptically inoculated into the culture medium, the top was sealed with liquid paraffin and incubated in the light (Rodina, 1972). Under constant illumination at room temperature growth was recorded in all the samples after 20-30 days of incubation. The bacteriochlorophyll was estimated in the logarithmic period which varied 20-30 days in different months during the study period and the period was found to be temperature dependent.

Estimation of Bacteriochlorophyll : 10 ml of culture samples were filtered through GFC filter and the filter was dissolved in 90% acetone, centrifuged and different wave lengths were measured in spectrophotometer and Bachl 'a', 'c', 'd' were estimated according to the procedure given by Strickland and Parsons (1968).

Anoxic waters often contain both green sulfur bacteria and organisms with chlorophyll 'a' (Chl 'a') of algae which sink from oxic surface waters and cyanobacteria which grow there (Cohen, 1984). When chlorophyll 'a' is also present, the problem of calculating the standing stock of green sulfur bacteria becomes more difficult, because the absorbance peak of green sulfur bacterial chlorophylls (Chl-GSB) is very close to that of chlorophyll '*a*' *i.e.* 663 nm (Lorenzen, 1967). Equations developed by Parkin and Brown (1981) for mathematically separating chlorophyll '*a*' from Chl-GSB in

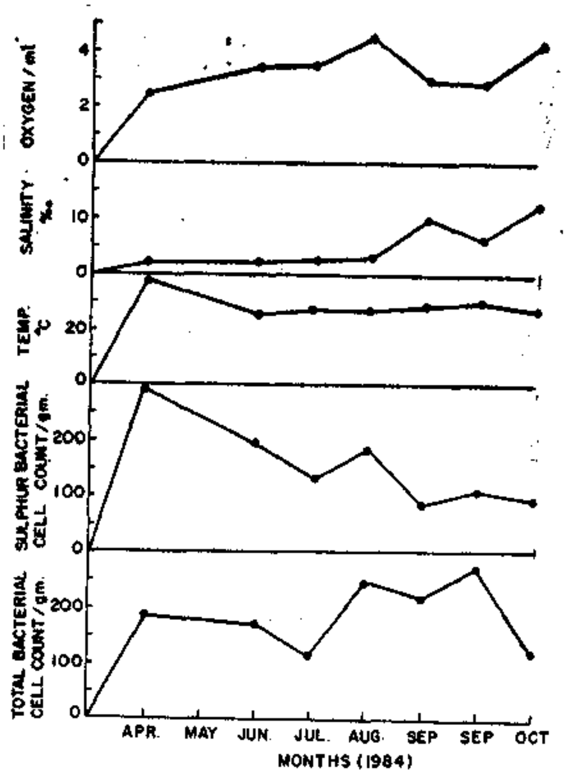

Fig. 2. Bacterial and environmental parameters in the microaerophilic region of mangrove (*Acanthus* Bed).

acetone extracts works only if no decomposition products of Chl-GSB are present. Upon decomposition (from chlorophyll to pheopigment) the absorbance of Chl-GSB shifts from 653 nm to near 658 nm (Gloe *et al.*, 1975), so that Parkin and Brown's equation would over estimate the concentrations of chl 'a'. Most systems with green sulfur bacteria probably contain significant quantities of these bacterial pheopigments (Pheo-GSB).

Calculating pigment concentrations in cultures containing Chlorophyll 'a', Pheo-GSB and Chl-GSB which absorb in acetone at 663, 658 and 653 nm is difficult, because of their close absorbances. Determination of chlorophyll 'a' or green bacterio-chlorophyll can therefore be difficult in anoxic cultures. Recently Caraco (1986) developed equations for mathematically separating Tchl-GSB from Tchl 'a' from the shapes of absorbance peaks of acidified 90% acetone extracts.

Suboxic condition existed in March and Apri and anoxic condition prevailed in all the other months (Table 1). Mixed cultures of chlorobacteriaceae developed in culture flask under conditions at RT after 20-30 days incubation were identified as *Chloronema*, *Chromatium*, *Beggiatoa*, *Thiopedia* and *Leucothiobacteria*. Unidentified brown chlorobacteriaceae were also present.

The pH of the Green sulphur bacterial culture medium during inoculation ranged from 7.2-7.6, but after 25 days of incubation due to intense growth of GSB the pH came

TABLE 1. *Total anaerobes, sulphur-reducers and hydrographic data during March-October*, 1984

Month	Total anaerobes 10^6/gm	Sulphur reducers 10^3/gm	Temp. °C	Sal. ‰	O_2 ml/lit.	Sediment pH	Sediment Eh mv	H_2S mg/lit.
March	184	290	30.7	1.79	2.43	7.4	—122.00	45
June	142	196	25.6	1.84	3.33	7.8	—167.00	40
July	144	86	26.4	1.29	3.21	8.0	—185.00	34
July	88	164	25.7	0.82	2.33	8.05	—55.00	44
August	244	175	26.6	2.77	4.30	8.2	—85.00	36
September	224	88	28.3	9.33	2.85	8.3	—55.00	35
September	260	102	29.1	5.92	2.75	8.0	—65.00	39
October	124	86	27.5	12.45	4.15	8.0	—55.00	42

RESULTS

The temperature ranged from 25.6°C in June to 30.7°C in April, 1984. Salinity varied between 0.82‰ in July to 12.45‰ in October. Oxygen followed the same pattern as salinity, the lowest being recorded in July and highest in October (Fig. 2).

The pH of the mangrove sediment samples ranged from 7.4 in March 1984 to 8.3 in September 1984. Whenever Eh decreased pH was found to increase establishing an inverse relationship between Eh and pH. Eh varied between —55 mv during July, September and October, 1984 to —185 mv during July, 1984. The redox potential revealed seasonal variation which influenced the whole sediment profile.

down to 3.0 and varied between 3.0-3.8. On gram-stained slides, the culture appeared to consist of gram-negative, non-motile rods of spherical cells ranging in size between 0.6 μ to 1.5 μ. The sulphide was oxidised anaerobically by *Leucothiobacteria* into sulphur and deposited externally as white powder in the medium (Plate I). Hydrogen Sulphide was estimated by the method from FAO Fisheries Manual and the H^2S ranged from 34-45 mg/litre after 25 days of incubation (Table 2).

Maximum development of bacteriochlorophyll was found during April 1984. Bachl 'a' was encountered in July 1984, whereas Chl 'a' and Bachl 'c' and 'd' were more in April (Fig. 3). *Rhodothiobacteria*, *Chlorobacteria* and *Leucothiobacteria* were isolated from all the sediment samples during March-October, 1984.

Discussion

Dense populations of anaerobic bacteria were found in the surface sediments ranging from 260 × 10⁸/gm during September 1984 to 88 × 10⁸/gm during July 1984. Sulpur bacteria ranged from 290 × 10⁸ to 88 × 10⁸. The occurrence of green sulfur bacteria and purple sulfur bacteria was indicated by green and purple colour produced after 20-30 days incubation period. Baas Becking (1925) found green bacteria in brackishwater, but not in brine. In the present study GSB and PSB were encountered invariably in all the sediment samples throughout the study period which indicated the presence of these bacteria in anoxic mangrove environment and the availability of sulfate in these environments.

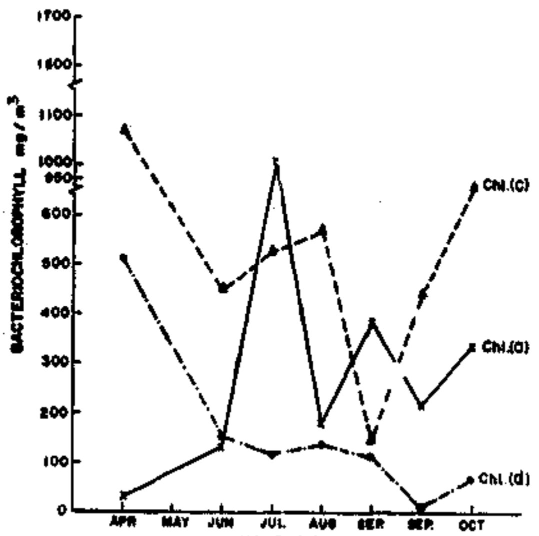

Fig. 3. Bacteriochlorophyll characteristic of phototrophic thionic bacteria in the micro-aerophilic region near Cochin.

Van Niel (1931) pointed out, bacteriochlorin, the green pigment of *Chlorobium limmicola* differs from the chlorophyll of algae. Pigment analysis by Spectrophotometric techniques revealed the structure and relative biomass abundance of the photoautotroph from the anaerobic cultures of present investigation.

Maximum bacteriochlorophyll 'a' (1650.6 mg/m⁻³) was encountered in May, whereas bacteriochlorophyll 'c' and 'd' were found more during April 1984 (1069.2 mg/m⁻³ and 510 mg/m⁻³ respectively) (Table 2). According to Ellis (1932) *Leucothiobacteria* includes all of the colourless sulfur bacteria. All thionic, achromic bacteria which deposit sulfur intracellularly are classified under *Leucothiobacteria* in the present study (Fig. 1).

The main factors determining the growth of Green Sulphur bacteria in the laboratory were light and high sulphide concentration, whereas the growth of purple sulphur bacteria was mainly controlled by subdued light and low sulphide concentration. Purple Sulfur bacteria gave the anoxic cultures a pinkish orange colour. But absorbance spectra of acetone extracts of particles from the coloured cultures showed that purple sulfur bacteria were not present in large numbers (as there was no absorbance at 772 nm). This indicated that the colour of the culture was probably due to unidentified brown coloured green sulfur bacteria (Gloe *et al.*, 1975; Pfennig, 1967).

Also, the green pigments developed in the present study did not show maximum absorption at 690 wave length. However, maximum absorption was recorded at approximately 662 wave length. Based on these results the predominant genus can be identified as *Chlorobium* and the chlorophyll of this strain is named as *Chlorobium chlorophyll* (Larsen, 1953) formerly called as *bacterioviridin* (Metzner, 1922).

Stainer and Smith (1960) discovered two types of '*Chlorobium chlorophyll*' designated as *Chlorobium chlorophyll* '650' and *Chlorobium chlorophyll* '660' according to their absorption maximum in solution. The present pigment can be designated as *Chlorobium chlorophyll* '660' since it showed absorption maximum at 662 nm. The flora may be *Chlorobiineae* containing bacteriochlorophyll of 'c' series (=*Chlorobium chlorophyll* 660 series). Parkin and Brown (1981) calculated the biomass of photosynthetic bacteria by estimating the Bchl (Bchl-GSB) in a meromictic lake measured from the absorption of 90% acetone extracts of particles, out of which 40% of the pigment was algal, but in the present observation the estimated level of pigments will be entirely from GSB as it is cultured anaerobically in a liquid medium and as such alga cannot exist in anaerobic medium.

The enormous amount of chlorophyll 'a' encountered in April (Table 2) may be due to high concentration of bacteriochlorophyll (Chl-GSB) or the presence of pheopigments of Chl-GSB which has been neglected in Parson and Strickland's equation which has lead to errors in calculated concentrations of bacterial pigments particularly in anoxic cultures. The chlorophyll 'a' recorded in the present study was actually bacteriochlorophyll (Chl-GSB), but after decomposition (from chlorophyll to pheopigment) the absorbance of Chl-GSB shifts from near 653 nm to near 658 nm (Gloe *et al.*, 1975) so that equations would over-estimate the concentrations of Chl 'a'.

The increase in the total anaerobic bacterial cell count (Fig. 2) in the postmonsoon months was indicative of the abundance of organic matter and their maximum rates of uptake. High counts of anaerobes can be attributed to high concentrations of phosphates and nitrates which results from detritus degradation, rain-fall, etc. Consistent number of bacteria in the mangrove soil in monsoon months reflected the stability of nutrient level in the soil. Temperature and oxygen showed direct relationship with anaerobic count in monsoon months whereas in postmonsoon months also total anaerobic counts indicated negative relationship with oxygen. ZoBell (1946) reported that the distribution of anaerobes in marine environment was not related to oxygen concentration. Gundersen *et al.* (1972) pointed out that the amount of available organic nutrients in the environment was the only single factor controlling the distribution of anaerobic bacteria.

Reduced Eh recorded (Table 1) in the later part of July 1984 may be due to reduced amounts of carbonaceous substrates in the area sampled or due to low tide water level. The rate and magnitude of the increase in reducing power varies with the substrate added to the mangrove. Only sulphur reducing microorganisms cause the change of Eh through consumption of O_2 and the liberation of reduced products. The milieu of the sediments has been delimited by ZoBell (1946). He gives the Eh limits as from +350 mv—500 mv and the pH limits as from 6.4 to 9.5. ZoBell (1946) states that bottom deposits are rich in organic matter and bacteria are generally reducing. Negative Eh values or reducing conditions are also the property of fine sediments, coarser sediments being less reducing. Eh values reduced with depth and high Eh and chlorophyll 'a' value during March, June, July may be attributed to the surface sampling of the clayey mud. Also sufficient available carbohydrates are present in the mud, most of the oxygen will be utilised for decomposition process before it penetrates too deeply in the liquid-mud layer. Bass Becking and Wood (1955) characterised true sulphide muds only if it is found to have a very low Eh which may be —270 mv to —300 mv. In the present observation —185 was the highest recorded Eh during July 1984 indicating anaerobic and microaerophilic conditions. Sulphur bacteria ranged from 4.2-19.4% of the anaerobes isolated.

The Eh is controlled on the positive side by photosynthesis and respiration and organic reducing biological systems on the negative. When colloidal iron sulphydryl and free hydrogen sulphide occur in sediments due sulphur-reducing bacterial actior. the sediment Eh may fall to —100 mv. The fall in Eh may be due to reducing effect of decomposing detritus and also due to liberatior of phosphoric acid from the ferric phosphate in the mangrove sediment. Debyser (1952) showed little correlation between Eh, particle size and the number of microbes, but found that the Eh depends on the history of the sediment *i.e.* newly formed sediments tend to have a low Eh and high SRB content.

Bass Becking *et al.* (1957) divided marine environments based on chemical reactions of increasing and decreasing pH and Eh into limestone waters, oligotrophic waters, estuarine sediments, estuarine water, geothermal regions and evaporites. Eventhough the sampling was done in marshy mangrove area the pH and Eh ranges of Karuthedam mangrove sediments fell under the ranges of *estuarine sediments* as the pH ranged between 7.0-8.3 and Eh —55 to —185 mv as in the estuarine sediments observed by Bass Beeking *et al.* (1957) (pH ranges between 5.3-9.4 and Eh +600 mv to 350 mv).

Faculative sulphate reducers were present in micro-aerophilic conditions during July and September which was indicated by reduced Eh and increased pH. Micro-aerophilic conditions prevailed during these months in the sampling area harbouring 260×10^{6}/gm of total anaerobes and 164×10^{8}/gm of sulphur reducing bacteria and oxygen value never exceeded 2.85 ml/litre in these months, indicating microaerophilic state in this area. The exudates of *Acanthus ilicifolius, Avicinia officinalis* growing in sulfate rich marshy soils may ultimately do harm because they serve as sources of energy to sulfate-reducing anaerobes. When the exudation and available carobhydrate is intense, the level of H_2S may be so great to kill the indigenous plants. The H_2S produced by sulphate-reducing bacteria may also be the cause of death of beneficial nematodes and fungi present in the mangrove ecosystem. Thionic microbes that reduce the availability of sulfate have a profound influence on mangrove soil fertility because they diminish the supply of major sulfur source for the phytoplankton and the higher crops. Beyond this fact, however, sulfate reducing bacteria can have a great economic influence as low concentrations of the product of their metabolism are highly toxic to fishes and prawns during low tide in mangrove ecosystem.

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